Modulatory Effect of Dieckol on Markers of Cell Proliferation, Apoptosis, Invasion, Metastasis and Angiogenesis

Chapter - III
MODULATORY EFFECT OF DIECKOL ON MARKERS OF CELL PROLIFERATION, APOPTOSIS, INVASION, METASTASIS AND ANGIogenesis

INTRODUCTION

Cancer arises as a result of changes in the genes leading to the attainment of six hallmark capabilities including self sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis. Changes in the expression of molecules that regulate cell cycle and cell proliferation pathways are disturbed in malignant cells with resulting loss of control of cell proliferation (Diaz-Cano, 2008; Hanahan and Weinber, 2011). PCNA functions as auxiliary protein for DNA polymerase δ which is required for DNA synthesis and cell cycle progression. Furthermore, the rate of PCNA synthesis is linked with the proliferative rate of cells (Stoimenov and Helleday, 2009; Strzalka and Ziemienowicz, 2011). Transcription factor nuclear factor-κB (NF-κB) regulates the expression of a wide variety of genes involved in cellular events such as inflammation, immune response, proliferation, apoptosis and cancer invasion (Chaturvedi et al., 2011).

Apoptosis evasion is recognized as a prerequisite for cancer cell to acquire permissive environment for angiogenesis, invasion and metastasis (Diaz-Cano, 2008; Hanahan and Weinber, 2011). Increasing evidence from epidemiological, preclinical and clinical studies provide evidence that dysregulated inflammatory response plays a pivotal role in a multitude of chronic ailments including cancer (Plati et al., 2011). The molecular mechanism(s) by which chronic inflammation drives cancer initiation and promotion include increased production of pro-inflammatory mediators, such as cytokines, chemokines, reactive oxygen intermediates, increased expression of oncogenes, COX-2 (cyclo-oxygenase-2), 5-LOX (5-lipoxygenase) and MMPs (matrix metalloproteinases), and pro-
inflammatory transcription factors such as NF-κB (nuclear factor κB), that mediate tumour cell proliferation, transformation, metastasis, survival, invasion, angiogenesis (Ben-Neriah and Karin, 2011; Luqman and Pezzuto, 2010). Still the putative research have not been initiated to validate the effect of dieckol on molecular markers of cell proliferation, apoptosis, invasion, metastasis and angiogenesis during hepatocarcinogenesis.

**MATERIALS AND METHODS**

**Drug and Chemicals**

Dieckol, N-nitrosodiethylamine, 3,3-diaminobenzidine, diethylpyrocarbonate (DEPC) and Trizol were purchased from Sigma Chemical Company, St. Louis, MO. Antibodies against PCNA, NF-κB, Bcl-2, Bax, caspase-3, VEGF, MMP-2 and MMP-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against β-actin was obtained from Sigma. Horse radish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz, CA. All other chemicals and reagents used were of analytical grade.

**Maintenance of animals and Tumor induction**

Protocol as same as that of second chapter in order to avoid the redundancy the methodology has not been repeated here.

**Isolation of dieckol**

Protocol as same as that of first chapter in order to avoid the redundancy the methodology has not been repeated here.

**Processing of blood and tissue samples, Dieckol preparation and Experimental design**

Protocol as same as that of second chapter in order to avoid the redundancy the methodology has not been repeated here.
Western blotting analysis and RTPCR

Protocol as same as that of first chapter in order to avoid the redundancy the methodology has not been repeated here.

Immunohistochemical analysis

The glass section slides were dewaxed and rehydrated through a gradual decrease in ethanol concentration. The slides were incubated in sodium citrate buffer (pH 6.0) for two cycles of 5 minutes in a microwave oven for antigen retrieval. After washing with PBS, the tissue section slides were then treated with 3% hydrogen peroxide to remove any endogenous peroxidases. Blocking was performed at 4°C using normal goat serum in a humidified chamber for several hours. The sections were then incubated overnight at 4°C in a humidified chamber using various primary antibodies: rabbit polyclonal antibodies for NF-κB, COX-2 and mouse monoclonal antibodies for VEGF, MMP-2 and MMP-9. The slides were washed with TBS and then incubated with anti-rabbit and anti-mouse biotin-labelled secondary antibody (Dako, Carprinteria, CA, USA) followed by streptavidin-biotin-peroxidase for 30 minutes each at room temperature. The samples were washed with PBS and the immunoprecipitate was visualized by treating with 3, 3'-diaminobenzidine (Dako, Carprinteria, CA, USA). After 25min, the slides were counterstained with hematoxylin and the brown color signifying the presence of antigen bound to antibody was detected by light microscopy. For negative controls, the primary antibody was replaced with TBS. Positive controls for each antibody were also processed simultaneously. Specificity of staining was controlled by omission of primary antibody that resulted in absence of staining. The results were expressed as the percentage of immunopositive cells.
**Statistical analysis**

Data were analysed by one way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) using a statistically software package (SPSS for Windows, V. 13.0, Chicago, USA). Results were presented as mean ± S.D. p-values < 0.05 were considered as statistically significant.

**RESULTS**

Figure 16 represents the western blot data for PCNA, Bcl-2, Bax and caspase-3. A significant increase in the expression of PCNA and Bcl-2 with decrease in the expression of Bax and caspase-3 were found in NDEA administered animals (group-3) compared to control (group-1). Treatment (group-4) of dieckol to NDEA treated animals significantly decreased the expression of PCNA and Bcl-2 and increased the expression of Bax and caspase-3 compared to NDEA treated animals (group-3). Administration of dieckol alone (group-2) did not significantly affect the expression of the markers analyzed compared to control.

Figures 17A to 17E and 18 represent the immunohistochemical and mRNA expression of NF-κB (p65) and COX-2, VEGF, MMP-2 and MMP-9 in control and experimental animals. A significant increase in the expression of NF-κB (p65) and COX-2, VEGF, MMP-2 and MMP-9 was found in NDEA administered animals (NDEA) compared to control (Control). Administration of dieckol to NDEA treated animals significantly decreased the expression of NF-κB (p65) and COX-2, VEGF, MMP-2 and MMP-9 to NDEA treated animals. Administration of dieckol alone (group-2) did not significantly affect the expression of NF-κB (p65) and COX-2, VEGF, MMP-2 and MMP-9 compared to control.
Figure 16: The effect of dieckol on PCNA, Bcl-2, Bax and caspase-3 expression in the liver tissue of control and experimental animals

36kDa PCNA

26kDa Bcl2

23kDa Bax

32kDa Caspase-3

43kDa β actin

* Significantly different from group 1 (p<0.05) ANOVA followed by LSD
** Significantly different from group 1 (p<0.001) ANOVA followed by LSD
★ Significantly different from group 3 (p<0.001)
Figure 17A: Immunohistochemical staining of NF-κB in the liver tissue of control and experimental animals.

Immunohistochemical staining of NF-κB in the liver tissue of control and experimental animals (mean ± SD; n=6)
Figure 17B: Immunohistochemical staining of COX-2 in the liver tissue of control and experimental animals

Immunohistochemical staining of COX-2 in the liver tissue of control and experimental animals (mean ± SD; n=6)
Figure 17C: Immunohistochemical staining of VEGF in the liver tissue of control and experimental animals

Immunohistochemical staining of VEGF in the liver tissue of control and experimental animals (mean ± SD; n=6).
Figure 17D: Immunohistochemical staining of MMP-2 in the liver tissue of control and experimental animals

Immunohistochemical staining of MMP-2 in the liver tissue of control and experimental animals (mean ± SD; n=6).
Figure 17E: Immunohistochemical staining of MMP-9 in the liver tissue of control and experimental animals

Immunohistochemical staining of MMP-9 in the liver tissue of control and experimental animals (mean ± SD; n=6).
Figure 18: The effect of dieckol on NF-κB, COX-2, VEGF, MMP-2 and MMP-9 expression in the liver tissue of control and experimental animals.

715bp NF-κB (p65) 209bp COX-2

576bp VEGF 249bp MMP-2

753bp MMP-9

350bp β-actin

*Significantly different from group 1 (p<0.001) ANOVA followed by LSD, †Significantly different from group 3 (p<0.01) (Mean ± SD; n=6).
DISCUSSION

NDEA usually causes genomic damage in exposed cells. As a consequence, the damaged cells may be triggered to proliferate with genomic damage, leading to the formation of cancerous cells that showed apoptosis evasion, increased cell proliferation, angiogenesis, invasion and metastasis (Hanahan and Weinberg, 2011). In the present study demonstrated that dieckol was capable of inducing apoptosis and inhibiting cell proliferation, angiogenesis, invasion and metastasis which result in suppression of hepatocarcinogenesis induced by administration of NDEA.

Several studies have demonstrated that inhibition of cell proliferation creates environment of cancer cell to undergo apoptosis (Thompson et al., 2004). Apoptosis evasion in NDEA-induced hepatocarcinogenesis was associated with imbalance in pro-apoptotic and anti-apoptotic proteins together with downregulation of caspases (Taha et al., 2010; Khan et al., 2011). Our results are consistent with the upregulation of anti-apoptotic proteins and downregulation of pro-apoptotic proteins and caspases which prevents cells from undergoing apoptosis through interference with the mitochondrial signaling pathway reported in various malignant tumours (Diaz-Cano, 2008; Stoimenov and Helleday, 2009; Strzalka and Ziemienowicz, 2011). Dieckol administration triggered mitochondrial mediated apoptosis in NDEA treated animals. This results in activation of pro-apoptotic proteins such as Bcl2-associated X protein (Bax) that execute their pro-apoptotic effects by directly binding and caspase-3 and inactivating anti-apoptotic Bcl-2 proteins.

Our results are consistent with previous reports about the induction of apoptosis by dieckol in various human cancer cell lines and animal tumour models has been reported to be associated with
increased expression of the pro-apoptotic protein Bax and a reduction in the anti-apoptotic proteins Bcl-2 (Zhang et al., 2011; Oh et al., 2011; Lee et al., 2012).

Dysregulation of apoptosis increase susceptibility to enhanced cell proliferation and cell survival (Diaz-Cano, 2008; Hanahan and Weinber, 2011). The regulations of apoptosis involve a dynamic interplay of several molecules. Among these, the release of Bcl-2 family proteins such as Bcl-2, and Bax are the major regulators of apoptosis, while caspases are the major executioners (Chaturvedi et al., 2011).

Apoptosis evasion and the rapid growth of cancerous cells within a tumour are often complemented by the development of vascularisation (angiogenesis) crucial for local progression and development of distant metastasis in malignant tumour (Diaz-Cano, 2008; Hanahan and Weinberg, 2011). The administration of the carcinogen, NDEA, caused upregulation of NF-κB that binds to DNA and results in transcription of genes that contribute to tumorogenesis, such as cell proliferation, inflammatory, antiapototic and positive regulators of cell proliferation and angiogenesis (Sivaramakrishnan and Devaraj, 2010; Khan et al., 2011). Our results supported that activation of NF-κB signaling cause phosphorylation of IκB, which triggers ubiquitination and subsequent degrading and activating of NF-κB. Treatment of dieckol-mediated blockage of IκBα degradation in hepatocellular carcinoma can reduce the nuclear content of NF-κB thereby, dieckol prevents NF-κB’s binding activity.

In addition, dieckol suppressed various NF-κB-regulated gene products including vascular endothelial growth factor (VEGF), the proinflammatory enzyme cyclooxygenase 2 (COX2), matrix metalloproteinase-2 and matrix metalloproteinase-9 (MMP-2 and MMP-9) which are associated with tumor progression and metastasis (Zhang et al., 2011). Several studies on human cancer and animal
tumour models have documented that increased tumor expression of COX-2, VEGF, MMP-2, and MMP-9 was correlated with more aggressive lesions that sustains inflammation, tumour growth, progression and metastasis (Noriyuki et al., 2007; Liao et al., 2010). Consistent with findings from literature, angiogenic effects of VEGF seen in the present study are also mediated via the upregulation of MMPs that degrade the components of the extra cellular matrix (ECM) during metastatic dissemination of tumour cells and thus promote tumour invasion and metastasis (Small et al., 2008). However, activation of NF-κB can convert inflammatory stimuli into tumor growth signals that are mediated by cytokines, chemokines, prostaglandins, nitric oxide and leucotrienes, which contribute to tumor promotion by altering normal cellular signaling cascades (Basseres and Baldwin, 2006). These prostaglandins play an important role because they can affect cell proliferation, mitosis, cell adhesion, apoptosis, and immune surveillance (Morrison, 2011).

NDEA induced COX2 expression causes arachidonic acid conversion into prostaglandins and are able to activate the procarcinogenesis. It has been suggested that COX2 induction is mediated by the NF-κB signaling pathway (Sivaramakrishnan and Devaraj, 2009). Dieckol treated groups have been shown to downregulate the COX2 expression, thereby maintaining increased levels of free arachidonic acid in the cells, which might have resulted in the suppression of carcinogenesis. Downregulation of NF-κB, VEGF, and MMPs and by inflammation, dieckol seen in the present study was correlated with the inhibition of tumor angiogenesis, invasion and metastasis resulting in suppression of liver tumour induced by NDEA. These findings thus raise a cautionary note about dieckol that inhibits angiogenesis, invasion and metastasis in cancer therapy.

In conclusion, our data showed that dieckol could induce mitochondrial-dependent pathway of apoptosis by altering the ratio of pro-apoptotic and anti-apoptotic mediators, and activation of
caspases. Downregulation of NF-κB, COX-2, VEGF, MMP-2 and MMP-9 by dieckol provide evidences that dieckol could act as a legitimate agent to inhibit cell proliferation, inflammation, angiogenesis, invasion and metastasis and induce apoptosis in cancer chemoprevention.